PLANT POPULATION DENSITY EFFECTS ON THE ALKALOID, SOLANESOL, AND CHLOROGENIC ACID CONTENT OF BURLEY TOBACCO[1]

By W.S. SCHLOTZHAUER[2], M.J. KASPERBAUER[3], and R.F. SEVERSON[2]

Genetically-uniform burley tobacco (*Nicotiana tabacum* L.) plants were grown in three field population densities (6,400, 22,000, and 111,000 plants/ha) to determine effects on concentrations of nicotine and minor alkaloids in growing tobacco and alkaloids, solanesol, and chlorogenic acid in air-cured tobacco. Alkaloid and solanesol concentrations were lowest in close-spaced and highest in wide-spaced plants; whereas, chlorogenic acid concentrations were highest in close-spaced and lowest in wide-spaced plants. Pyrolysis studies indicated that the ratio of tar to nicotine decreased with wider plant spacing.

Key words: *Nicotiana tabacum*, plant spacing, leaf chemistry, pyrolyzate.

INTRODUCTION

Agriculturalists occasionally modify field plant population densities to facilitate new production procedures, such as those better suited to mechanization. When using non-traditional plant spacing, however, it is important to evaluate potential effects on modification of the plant products. Effects on leaf composition are particularly important to evaluate potential effects on modification of the plant products. Effects on leaf composition are particularly important for tobacco (*Nicotiana tabacum* L.) and for other leaf crops. Papenfus (12) stressed the importance of understanding the relationship between plant population density and tobacco leaf composition.

The physical and chemical properties of tobacco are regulated by genetics and by total growth environment (4). Numerous studies have involved effects of cultivar, nitrogen nutrition, and other production practices on tobacco yield and alkaloid content (2,3,17,19). However, little information is available on population density effects on spectral distribution of light in the growing canopy and effects on growth, development, and chemical composition of individual tobacco plants.

Research with various plant species has shown that green leaves absorb most of the red light from sunlight while reflecting or transmitting most of the far-red light (11). Consequently, a plant surrounded by many green leaves (each reflecting far-red light would receive a higher ratio of far-red relative to red light (8). Conversely, a plant in a wide-spaced population would receive a lower far-red/red light ratio. The far-red/red light ratio received by a plant is of particular importance because it acts through the phytochrome system within the plant to regulate many physiological processes during growth and development (1).

Under a controlled environment, the far-red/red light ratio (acting through the phytochrome system) can regulate chloroplast ultrastructure (9), and chlorophyll a/b ratio, leaf morphology, and photosynthetic efficiency (10). A low far-red/red light ratio (as would occur in wide-spaced field populations) results in plants characterized by short stalks, thick leaves, and large root systems (6), and by high nicotine and low soluble phenolic contents (18). We hypothesized that this natural bioregulation of developmental processes is involved in tobacco population density effects on leaf composition of field grown tobacco. The objectives of the present study were (a) to determine the relationship between field population density and alkaloid, solanesol, and chlorogenic acid content of burley tobacco, and (b) to determine the effect of plant population density on tar/nicotine ratios in derived pyrolyzates.

---

[1] *Presented in part at the 41st Tobacco Chemists' Research Conference, Greensboro, N.C. October 1987.*

[2] *Tobacco Quality and Safety Research Unit, Agricultural Research Service, United States Department of Agriculture, Richard B. Russell Research Center, P.O. Box 5677, Athens, Ga. 30613.*

[3] *Agricultural Research Service, United States Department of Agriculture, Coastal Plains Research Center, Florence, S.C. 29502.*

[4] *Names of products are included for the benefit of the reader and do not imply endorsement of preferential treatment by USDA.*

Contribution received August 8, 1988, Tob. Sci. 33:47-52, 1989.

Table 1. Alkaloid Concentrations in Mid-Stalk Leaf Lamina at Topping and at Harvest.

Plant Spacing	Growth Stage	Alkaloid				
		Nicotine	Nornicotine	Anabasine	Anatabine	Total
Close	Topping	8.13±1.20[a]	0.13±0.03	0.06±0.04	0.16±0.04	8.48±1.26
	Harvest	12.54±2.24	0.19±0.05	0.06±0.01	0.28±0.08	13.07±2.38
Normal	Topping	10.62±0.33	0.15±0.01	0.05±0.01	0.23±0.02	11.04±0.35
	Harvest	30.34±2.35	0.74±0.12	0.16±0.01	1.17±0.16	32.29±2.53
Wide	Topping	16.51±0.67	0.28±0.03	0.08±0.01	0.41±0.04	17.77±0.51
	Harvest	50.68±4.51	1.05±0.10	0.19±0.01	1.82±0.15	53.60±4.69

[a]Values are means for three years ± SE.

MATERIALS AND METHODS

Tobacco Culture and Sampling. Burley tobacco (*Nicotiana tabacum* L. cv. KY 14) plants were grown in field plots near Lexington, KY, for three consecutive growing seasons (1980 through 1982). Each year a 15 m x 65 m plot of Maury silt loam (Typic Paleudalf) with a cover crop was plowed under several weeks before transplanting, and 260 kg/ha of nitrogen was broadcast as NH_4NO_3 and disked in just before the plants were set. Subplots for the different spacings were approximately 9 m x 9 m (close), 6.7 m x 27 m (normal), and 13 m x 13 m (wide). Within the respective plots, plant spacings were 'close' = 30 cm × 30 cm = 111,000 plants/ha, 'normal' = 45 cm × 100 cm = 22,000 plants/ha, and 'wide' = 125 cm x 125 cm = 6,400 plants/ha. The same procedure was followed each of the three years. The three years were used as replicates in statistical analysis of the data.

The same lot of inbred seed was used for all plots and all years in order to minimize genetic variability among plants. Each year, the seedlings were started and grown in individual containers, until attaining transplant size, in a controlled environment at 28 °C under 14-hr photoperiods at approximately 425 μmols m^{-2} s^{-1} from cool-white fluorescent lamps. This pre-transplant environment avoided premature floral induction which could have altered post-transplant field growth (7). Uniformly-sized seedlings were transplanted to field plots during the first week in June each year. Plants were irrigated as needed to avoid water stress during growth and development. Each year, plants were 'topped' (inflorescences and upper leaves shorter than 25 cm were removed) when approximately 25% of the plants had one or more open flowers. Axillary shoots (suckers) were removed by hand twice each week during the four-week period between topping and harvest. Suckers were removed by hand rather than chemically in order to avoid possible introduction of chemical residues that might complicate leaf constitutent determinations. After harvest, intact plants were air-cured for about six weeks within a conventional burley tobacco curing barn.

Mid-stalk leaf lamina samples were taken at topping (time of first flowering: 10 weeks after transplant) and at time of harvest. After conventional air-curing, lamina, midvein, and stalk samples were taken from top, middle, and bottom stalk positions for each of the three plant population densities. The top position was comprised of leaves 1, 2, and 3; the middle comprised of leaves 9, 10, and 11; and the bottom comprised of leaves 18, 19, and 20. (In this system of designating leaf numbers, leaf 1 was the top remaining leaf after the plant was topped). Each stalk position sample consisted of three leaves and the associated stalk segments from each of 10 stalks. The 10 stalks were randomly selected from each of the three population densities at each sampling time.

Leaf lamina, midvein, and stalk were separated immediately after sampling, quick-frozen, freeze-dried, and ground to pass a 40-mesh screen. Samples were stored in darkness, in air-tight freezer bags, at −40 °C prior to chemical analysis.

Alkaloid Analyses. The analytical determination of nicotine and the 'minor' tobacco alkaloids (nornicotine, anabasine, and anatabine) in leaf samples were performed using a modification of the method described by Severson *et al.* (16). Briefly, samples were extracted by sonification using 0.05 M methanolic potassium hydroxide, with known amounts of 2,4'-dipyridyl as internal standard for quantitation. Alkaloids were determined by glass capillary gas chromatography (GC2).

Solanesol and Chlorogenic Acid Analyses. Samples (25 mg) of air-cured plant parts were weighed in 2-ml reaction vials and 1-ml aliquots of a 50:50 (v/v) mixture of dimethylformamide/N, O-bis(trimethylsilyl)trifluoroacetamide (BSTFA, Pierce Chemical Co.)[4] containing 0.05% 1,3-dimyristin (internal standard) added. Vials were sealed, sonificated for 45 min, and placed in a 85 °C heater block for 30 min. Aftera centrifugation of the extracted residue, 1-μmL aliquots of the supernatant were analyzed by glass capillary gas chromatography equipped with a 0.54 mm x 15 m fused silica column containing SE-54 liquid phase and flame ionization detector. Column temperature programming from 100 °C to 330 °C at 8 °/min allowed quantitative determination of chlorogenic acid (27 min) and solanesol (35 min) with the internal standard eluting at 30 min.

Pyrolysis of Cured Leaf Samples. Mid-stalk samples were

Table 2. Summary of Statistical Treatment of Data in Table 1.

Variable	Alkaloid				
	Nicotine	Nornicotine	Anabasine	Anatabine	Total
Year (Y)	NS[a]	NS	NS	NS	NS
Plant Spacing (S)	**	**	**	**	**
Y x S	NS	NS	NS	NS	NS
Growth Stage (G)	**	**	**	**	**
S x G	**	**	**	**	**

[a]NS = not statistically significant at P=0.05, ** = significant at P=0.01.

Table 3. Alkaloid Concentrations in Air-Cured Tobacco Lamina, Midvein, and Stalk.[a]

Plant Spacing	Stalk Position	Nicotine	Nornicotine	Anabasine	Anatabine	Total Alkaloids
		Weight (mg/g dry weight)				
			Leaf Lamina			
Close	Top	5.00	0.21	0.05	0.08	5.34
	Middle	3.01	0.03	0.01	0.03	3.08
	Bottom	1.60	0.04	0.04	0.04	1.71
Normal	Top	20.49	0.11	0.12	1.17	21.89
	Middle	16.30	0.62	0.10	0.57	17.59
	Bottom	17.36	0.59	0.04	0.89	18.88
Wide	Top	40.30	0.99	0.23	1.46	42.98
	Middle	37.49	0.71	0.18	1.01	39.39
	Bottom	28.86	0.85	0.18	1.17	31.06
			Midvein			
Close	Top	0.93	<0.01	<0.01	<0.01	0.93
	Middle	0.40	<0.01	<0.01	<0.01	0.04
	Bottom	0.25	<0.01	<0.01	<0.01	0.25
Normal	Top	3.64	0.18	0.02	0.05	3.89
	Middle	2.38	0.18	0.02	0.02	2.60
	Bottom	2.18	0.18	0.02	0.03	2.41
Wide	Top	6.08	0.20	0.02	0.10	6.40
	Middle	6.04	0.26	0.04	0.08	6.42
	Bottom	5.81	0.30	0.03	0.14	6.28
			Stalk			
Close	Top	1.04	<0.01	<0.01	<0.01	1.04
	Middle	0.57	<0.01	<0.01	<0.01	0.57
	Bottom	0.31	<0.01	<0.01	<0.01	0.31
Normal	Top	3.70	0.07	<0.01	0.01	3.78
	Middle	2.03	0.05	<0.01	0.03	2.11
	Bottom	2.23	0.04	0.01	0.03	2.31
Wide	Top	4.76	0.13	<0.01	0.02	4.81
	Middle	4.94	0.09	<0.01	0.07	5.10
	Bottom	3.27	0.14	<0.01	0.09	3.50

[a] Samples composited for three growing seasons, three replicates each.

pyrolyzed using a platinum heating coil pyroprobe (Chemical Data Systems, Inc.) with the collection system described by Schlotzhauer et al. (14). Coil temperature was elevated to 700 °C for 10 sec in a stream of dry nitrogen. Such experimental parameters produced pyrolyzate with chemical component profiles similar to those obtained by mechanical smoking of cigarettes under conditions designed to simulate human smoking behavior. 'Tar', or total particular matter, was estimated by weighing the collection tube prior to, and immediately after, pyrolysis. Pyrolyzates were recovered by elution in acetone/chloroform (2:1, v/v) containing known amounts of 1-tetradecanol as internal standard. Reactive pyrolyzate components were converted to their trimethylsilyl ethers by reaction with BSTFA in sealed vials for 30 min at 76 °C. The silylation procedure was required to achieve resolution of nicotine from the numerous polar compounds produced during the pyrolytic breakdown of tobacco. Nicotine was quantified on a Hewlett-Packard model 5720A gas chromatograph equipped with 25 m x 0.3 mm fused silica column dynamically coated with SE-54 liquid phase, and flame ionization detection.

Statistical Treatment. Means ± standard errors (SE) and analysis of variance (ANOV) of the data were determined as outlined by SAS Institute (13).

RESULTS AND DISCUSSION

Nicotine and the 'minor' tobacco alkaloids were determined for burley tobacco leaf lamina from the mid-stalk positions samples at topping and harvest from the three population densities for three consecutive years. The same trends were evident each year. Mean values are shown in **Table 1**, and levels of significance are shown in **Table 2**. Alkaloid concentrations increased from time of topping to time of harvest. Increases were greatest in the wide-spaced and least in the close-spaced plant populations. At time of topping, consistently higher alkaloid concentrations were evident in wide-spaced plants, and by time of the harvest, plant density was a major determinant of alkaloid accumulation. These data are consistent with the observation (8) that number and nearness of competing plants can influence the amount of reflected far-red light and the far-red/red light ratio, which can regulate many aspects of plant development. It has been demonstrated (6,18) in controlled environments that a decreased far-red/red light ratio results in increased alkaloid accumulation and that the effects of a low far-red/red light ratio can be negated by immediate exposure to a high far-red/red light ratio. These observations strongly indicate that the phytochrome system within the plant can play a critical role in regulation of

Table 4. Summary of Statistical Treatment of Alkaloid Concentrations in Air-Cured Burley Tobacco Shown in Table 3.

Variable	Alkaloid				
	Nicotine	Nornicotine	Anabasine	Anatabine	Total
Plant Part (P)	***	**	**	**	**
Plant Spacing (S)	**	**	**	**	**
P x S	**	**	**	**	**
Stalk Position	*	NS	NS	NS	*

[a]NS = not statistically significant at P=0.05, * = significant at P=0.05, ** = significant at P=0.01.

Table 5. Solanesol in Air-Cured Burley Tobacco Leaf Lamina.

Plant Spacing	Stalk Position	Solanesol[a] (mg/g)
Close	Top	15.68±2.09
	Middle	11.02±1.56
	Bottom	9.30±0.74
Normal	Top	23.73±1.82
	Middle	11.05±1.48
	Bottom	10.01±0.11
Wide	Top	41.93±4.16
	Middle	23.43±2.39
	Bottom	20.26±1.14

[a]Means for three growing seasons ± SE. Values differed significantly at P=0.01 for both plant spacing and stalk position.

Table 6. Chlorogenic Acid in Air-Cured Burley Tobacco Leaves.

Plant Spacing	Stalk Position	Chlorogenic Acid[a] Lamina (mg/g)	Midvein
Close	Top	0.96±0.17	0.71±0.14
	Middle	0.80±0.06	0.57±0.05
	Bottom	0.92±0.17	0.51±0.07
Normal	Top	0.37±0.09	0.07±0.01
	Middle	0.76±0.03	0.32±0.01
	Bottom	0.77±0.05	0.36±0.03
Wide	Top	0.59±0.08	<0.05
	Middle	0.49±0.07	<0.05
	Bottom	0.40±0.01	<0.05

[a]Means for three growing seasons ± SE. Values for both lamina and midvein differed significantly at P=0.01 for plant spacing.

Table 7. Tar and Nicotine in Pyrolyzates of Air-Cured Tobacco.

Plant Spacing	Tar (mg/g)[a]	Nicotine (mg/g)[a]	Tar/Nicotine Ratio
		Leaf Lamina	
Close	214	3.32	64:1
Normal	322	19.12	17:1
Wide	336	34.61	10:1
		Midvein	
Close	326	1.35	241:1
Normal	314	3.64	86:1
Wide	305	6.05	50:1
		Stalk	
Close	450	1.17	385:1
Normal	461	2.41	191:1
Wide	452	2.82	160:1

[a]Composite of middle stalk positions from three growing seasons.

the developmental processes involved in formation of such secondary leaf components as alkaloids. The data in **Table 1**, having been obtained under field conditions over three growing seasons, are wholly consistent with the hypotheses based on the controlled-environment experiments.

After traditional air-curing, samples of differing stalk positions, plant parts, and population densities were analyzed for alkaloid concentration (**Tables 3 and 4**). Values for leaf lamina alkaloids at different stalk positions demonstrated increasing alkaloid accumulation in upper leaves, a trend observed for each population density although more pronounced in close-spaced tobacco than in the other field population density plants. Lower parts of close-spaced plants receive decreased light intensity and, because green leaves absorb red and transmit much of the far-red light, a much higher far-red/ red light ratio as shown in a prevous study (6). Alkaloid content of midvein was consistent with the trend of increasing concentration with decreased plant population density. Within stalk samples, alkaloid accumulations were generally consistent with the data for lamina both with regard to plant population density and stalk position. These data suggest that alterations of light spectral characteristics associated with plant density may not only affect production of alkaloids but may also influence the active transport of these compounds within the growing plants. Phytochrome-mediated regulation of partitioning has been demonstrated in controlled environments (9,10) and in the field (6,8).

The C_{45} alcohol, solanesol, is of particular interest to phytochemistry, since this constituent is the most abundant naturally occurring isoprenoid component of solanaeceous plants (20) and produces breakdown products important to physiological and biological properties of tobacco leaf and smoke (15). Concentrations of solanesol in air-cured lamina are shown in **Table 5.** Concentrations in midvein and stalk were below limits of detection. Solanesol accumulation patterns in lamina were consistent with those observed for alkaloids.

Levels of chlorogenic acid, the principal caffetannin occurring in *Nicotiana tabacum*, are known to vary widely with genotype, cultivation, stalk position, and post-harvest treatment (17). Chlorogenic acid accumulations (**Table 6**) in air-cured lamina and midvein obtained at differing field populations follow a trend unlike that observed for alkaloids and solanesol, with largest concentrations occurring in close-spaced plants. Concentrations in stalk were below limits of detection. Burley tobacco varieties, including that utilized for these experiments, are generally characterized by relatively low caffetannin and relatively high alkaloid content when cultivated under conventional agronomic practices (17), and it is apparent from the data in **Table 6** that such characteristics may be altered by changes in field plant population densities.

Differences in levels and distribution of tobacco leaf components would be expected to result in alteration of smoke chemistry. Comparative studies by Ishiguro and Sugawara (5) of smoke obtained from cigarettes fabricated from all lamina or all midrib plant parts reported six times the amount of nicotine present in smoke of the former as compared to the latter test cigarettes. Using pyrolysis data (**Table 7**) obtained for the three plant densities from the composited mid-stalk position samples representing three growing seasons in this study, we determined likely effects of plant population densities on tobacco smoke nicotine and smoke 'tar' as estimated by pyrolyzate values. The ratio of nicotine content of pyrolyzate obtained from normal-spaced lamina to that from the corresponding midvein and stalk was consistent with cigarette smoke data (5). In general, nicotine transferred into pyrolyzates in the same concentration orders as were observed for the plant parts and plant population densities shown in **Table 3**. Less variation in 'tar' levels due to plant spacing or plant part was apparent from these data; however, tar to nicotine ratios differed among plant parts and decreased dramatically with wider plant-spacing as nicotine concentrations increased.

ACKNOWLEDGEMENTS

The authors wish to thank Robert M. Martin, Linda B. Smith, Brenda G. King, T. G. Sutton, and Woodrow Sanders for technical assistance in these studies.

LITERATURE CITED

1. Borthwick, H.A. The biological significance of phytochrome. In *Phytochrome*. (K. Mitrakos and W. Shropshire, Eds.) Academic Press, New York, NY. pp. 27-44. 1982.
2. Campbell, C.R., J.F. Chaplin, and W.H. Johnson. Cultural factors affecting yield, alkaloids, and sugars of close-grown tobacco. **Agron. J.** 74:279-283. 1982.
3. Campbell, J.S., J.F. Chaplin, D.M. Boyette, C.R. Campbell, and C.B. Crawford. Effect of plant spacings, topping heights, nitrogen rates, and varieties of tobacco on nicotine yield and concentration. **Tob. Sci.** 26:66-69. 1982.
4. Garner, W.W. **The Production of Tobacco**. McGraw Hill, New York, NY. 1951.
5. Ishiguro, S., and S. Sugawara. Studies on the composition of smoke components of lamina and mid-rib cigarettes of flue-cured tobacco leaves by the trimethylsilylation method. **Agric. Biol. Chem.** 42:407-410. 1978.
6. Kasperbauer, M.J. Spectral distributions of light in a tobacco canopy and effects of end-of-day light quality on growth and development. **Plant Physiol.** 47:775-778. 1971.
7. Kasperbauer, M.J. Effect of pretransplant light on post-transplant growth and development of tobacco. **Agron. J.** 65:447-450. 1973.
8. Kasperbauer, M.J. Far-red light reflection from green leaves and effects on phytochrome-mediated assimilate partitioning under field conditions. **Plant Physiol.** 85:350-354. 1987.
9. Kasperbauer, M.J., and J.L. Hamilton. Chloroplast structure and starch grain accumulation in leaves that received different red and far-red levels during develoment. **Plant Physiol.** 74:967-970. 1984.
10. Kasperbauer, M.J., and D.E. Peaslee. Morphology and photosynthetic efficiency of tobacco leaves that received end-of-day red or far-red light during development. **Plant Physiol.** 52:440-442. 1973.
11. Knipling, E.B. Physical and physiological basis for the reflectance of visible and near infra-red radiation from vegetation. **Remote Sensing Environ.** 1:155-159. 1970.
12. Papenfus, H.D. Some aspects of stress management in tobacco. **Recent. Adv. in rob. Sci.** 13:27-51. 1987.
13. Statistical Analysis System. SAS User's Guide: Statistics. Version 5 ed., 956 p., SAS Institute Inc., Raleigh, N.C. 1985.
14. Schlotzhauer, W.S., R.F. Arrendale, and O.T. Chortyk. The rapid pyrolytic characterization of tobacco leaf carbohydrate material. **Beitr. Tabakforsch.** 13:74-80. 1985.
15. Schlotzhauer, W.S., R.F. Severson, O.T. Chortyk, and M.E. Snook. Pyrolytic formation of polynuclear aromatic hydrocarbons from petroleum-ether extractable constituents of flue-cured tobacco leaf. **J. Agric. Food Chem.** 24:992-996. 1976.
16. Severson, R.F., K.L. McDuffie, R.F. Arrendale, G.R. Gwynn, and J.F. Chaplin. Rapid method for analysis of tobacco nicotine alkaloids. **J. Chromatog.** 211:111-121. 1981.
17. Tso, T.C. **Physiology and Biochemistry of Tobacco Plants.** Dowden, Hutchinson, and Ross, Stroudsburg, PA. 1972.
18. Tso, T.C., M.J. Kasperbauer, and T.P. Sorokin. Effect of photoperiod and end-of-day light quality on alkaloids and phenolic compounds of tobacco. **Plant Physiol.** 45:330-333. 1970.
19. Weybrew, J.A., and W.G. Woltz. Production factors affecting chemical properties of flue-cured leaf. Part IV. Influence of management and water. **Tob. Int.** 177(6):46-51. 1975.
20. Wooten, J.B. Direct detection of solanesol in tobacco by ^{1}H and ^{13}C magic angle spinning NMR. **J. Agric. Food Chem.** 33:419-425. 1985.